

reactions comprised 0.01 pmoles of heat-denatured, end-labeled hairpin DNA (with the unlabeled complementary strand also present), 1 pmole primer (complementary to the 3' arm) and 0.5 units of DNAP*Taq* (estimated to be 0.026 pmoles) in a total volume of 10µl of 10 mM Tris-Cl, pH 8.5, 50 mM KCl and 1.5 mM MgCl<sub>2</sub>. As indicated, some reactions had different concentrations of KCl, and the precise times and temperatures used in each experiment are indicated in the individual figures. The reactions that included a primer used the one shown in Figure 6 (SEQ ID NO:17). In some instances, the primer was extended to the junction site by providing polymerase and selected nucleotides.

Reactions were initiated at the final reaction temperature by the addition of either the MgCl<sub>2</sub> or enzyme. Reactions were stopped at their incubation temperatures by the addition of 8 µl of 95% formamide with 20 mM EDTA and 0.05% marker dyes. The T<sub>m</sub> calculations listed were made using the Oligo™ primer analysis software from National Biosciences, Inc. These were determined using 0.25 µM as the DNA concentration, at either 15 or 65 mM total salt (the 1.5 mM MgCl<sub>2</sub> in all reactions was given the value of 15 mM salt for these calculations).

Figure 9 is an autoradiogram containing the results of a set of experiments and conditions on the cleavage site. Figure 9A is a determination of reaction components that enable cleavage. Incubation of 5'-end-labeled hairpin DNA was for 30 minutes at 55°C, with the indicated components. The products were resolved by denaturing polyacrylamide gel electrophoresis and the lengths of the products, in nucleotides, are indicated. Figure 9B describes the effect of temperature on the site of cleavage in the absence of added primer. Reactions were incubated in the absence of KCl for 10 minutes at the indicated temperatures. The lengths of the products, in nucleotides, are indicated.

Surprisingly, cleavage by DNAP*Taq* requires neither a primer nor dNTPs (*see* Fig. 9A). Thus, the 5' nuclease activity can be uncoupled from polymerization. Nuclease activity requires magnesium ions, though manganese ions can be substituted, albeit with potential changes in specificity and activity. Neither zinc nor calcium ions

support the cleavage reaction. The reaction occurs over a broad temperature range, from 25°C to 85°C, with the rate of cleavage increasing at higher temperatures.

Still referring to Figure 9, the primer is not elongated in the absence of added dNTPs. However, the primer influences both the site and the rate of cleavage of the hairpin. The change in the site of cleavage (Fig. 9A) apparently results from disruption of a short duplex formed between the arms of the DNA substrate. In the absence of primer, the sequences indicated by underlining in Figure 6 could pair, forming an extended duplex. Cleavage at the end of the extended duplex would release the 11 nucleotide fragment seen on the Fig. 9A lanes with no added primer. Addition of excess primer (Fig. 9A, lanes 3 and 4) or incubation at an elevated temperature (Fig. 9B) disrupts the short extension of the duplex and results in a longer 5' arm and, hence, longer cleavage products.

The location of the 3' end of the primer can influence the precise site of cleavage. Electrophoretic analysis revealed that in the absence of primer (Fig. 9B), cleavage occurs at the end of the substrate duplex (either the extended or shortened form, depending on the temperature) between the first and second base pairs. When the primer extends up to the base of the duplex, cleavage also occurs one nucleotide into the duplex. However, when a gap of four or six nucleotides exists between the 3' end of the primer and the substrate duplex, the cleavage site is shifted four to six nucleotides in the 5' direction.

Fig. 10 describes the kinetics of cleavage in the presence (Fig. 10A) or absence (Fig. 10B) of a primer oligonucleotide. The reactions were run at 55°C with either 50 mM KCl (Fig. 10A) or 20 mM KCl (Fig. 10B). The reaction products were resolved by denaturing polyacrylamide gel electrophoresis and the lengths of the products, in nucleotides, are indicated. "M", indicating a marker, is a 5' end-labeled 19-nt oligonucleotide. Under these salt conditions, Figs. 10A and 10B indicate that the reaction appears to be about twenty times faster in the presence of primer than in the absence of primer. This effect on the efficiency may be attributable to proper alignment and stabilization of the enzyme on the substrate.

The relative influence of primer on cleavage rates becomes much greater when both reactions are run in 50 mM KCl. In the presence of primer, the rate of cleavage increases with KCl concentration, up to about 50 mM. However, inhibition of this reaction in the presence of primer is apparent at 100 mM and is complete at 150 mM KCl. In contrast, in the absence of primer the rate is enhanced by concentration of KCl up to 20 mM, but it is reduced at concentrations above 30 mM. At 50 mM KCl, the reaction is almost completely inhibited. The inhibition of cleavage by KCl in the absence of primer is affected by temperature, being more pronounced at lower temperatures.

Recognition of the 5' end of the arm to be cut appears to be an important feature of substrate recognition. Substrates that lack a free 5' end, such as circular M13 DNA, cannot be cleaved under any conditions tested. Even with substrates having defined 5' arms, the rate of cleavage by DNAP*Taq* is influenced by the length of the arm. In the presence of primer and 50 mM KCl, cleavage of a 5' extension that is 27 nucleotides long is essentially complete within 2 minutes at 55°C. In contrast, cleavages of molecules with 5' arms of 84 and 188 nucleotides are only about 90% and 40% complete after 20 minutes. Incubation at higher temperatures reduces the inhibitory effects of long extensions indicating that secondary structure in the 5' arm or a heat-labile structure in the enzyme may inhibit the reaction. A mixing experiment, run under conditions of substrate excess, shows that the molecules with long arms do not preferentially tie up the available enzyme in non-productive complexes. These results may indicate that the 5' nuclease domain gains access to the cleavage site at the end of the bifurcated duplex by moving down the 5' arm from one end to the other. Longer 5' arms would be expected to have more adventitious secondary structures (particularly when KCl concentrations are high), which would be likely to impede this movement.

Cleavage does not appear to be inhibited by long 3' arms of either the substrate strand target molecule or pilot nucleic acid, at least up to 2 kilobases. At the other extreme, 3' arms of the pilot nucleic acid as short as one nucleotide can support